Dissociation of embryonic kidney followed by re-aggregation as a method for chimeric analysis.

Cite as an Accepted Article, doi: 10.1007/978-1-61779-851-1_12

Dissociation of embryonic kidney followed by re-aggregation as a method for chimaeric analysis

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Summary:

This chapter presents three methods for re-constructing mouse foetal kidney tissue from simple suspensions of cells. These techniques are very useful for a number of purposes; (i) they allow the production of fine-grained chimaeras in which cell autonomy of mutations can be tested, (ii) they provide an environment allow the renal differentiation potential of stem cells to be assessed, and (iii) they are an excellent system in which to study the mechanisms of self-organization. Each of the methods described here begins with disaggregation of embryonic mouse kidneys, followed by re-aggregation and culture; the main differences are in the culture methods, each of which has advantages for particular purposes.

Key words:
Tissue engineering, Metanephros, Chimaera, Self-organization, Mosaic, Organ culture.

1. Introduction

The ability to construct 'embryonic kidney tissue' by re-aggregation of initially-dissociated renogenic cells adds a powerful weapon to the armoury of kidney culture techniques. As well as uncovering basic processes of anatomical self-organization, the system allows experimenters to make fine-grained chimaeras that place 'test' cells in the context of developing kidney tissue. This provides a convenient way to assess the abilities of these cells to differentiate into various renal cell types, or their abilities to interfere with or promote the differentiation and morphogenesis of the host tissue. Formation of an embryonic kidney tissue from simple suspensions of cells is also a promising technique for stem cell-based tissue-engineering techniques in regenerative medicine, but these applications are beyond the scope of this article.

Here, we present two broadly similar methods for disaggregating embryonic mouse kidneys to obtain renogenic cells and then re-aggregating them to form organotypic tissue, with or without the addition of other cells to make chimaeras. One method was developed in Edinburgh, the other in Brisbane; both were published in 2010 (1;2) and both were highlighted by editorials in their respective journals (3;4). The methods each begin with enzymatic dissociation of kidneys, followed by random re-aggregation of cells using centrifugation. They then use conventional organ culture of the resulting aggregate to allow renal structures to re-form. Both have been used for published studies on the fates of exogenous cells in chimaeric re-aggregates (2;5). The Edinburgh method uses a temporary drug treatment to promote cell survival, while the Brisbane method uses a feeder layer of Wnt-secreting support cells.

Simple re-aggregations of the type described above result in the re-formation of ureteric bud epithelia and the induction of nephron epithelia from mesenchyme, followed by eventual connection of the nephrons to the ureteric bud tubules in the usual manner. At high magnification, there is little apparent difference between the tissues and those of an embryonic kidney that developed *in vivo*, but on low-power examination there is one very obvious difference: a normal embryonic kidney is arranged around a single, branched ureteric bud/ collecting duct tree that leads back to a single ureter, whereas in the re-

aggregates many small, disconnected ureteric bud 'tree-lets' form. This difference prevents the development of the normal cortico-medullary axis of the kidney, and would be a major handicap to clinical use since the drainage purpose of the collecting ducts is defeated and the urine-concentrating function of cortico-medullary organization is also missing. A very recent development of the Edinburgh method, which uses two sequential rounds of disaggregation and re-aggregation, results in a re-aggregate that is arranged around one single ureteric bud (Ganeva, Unbekandt, Davies in press, ref details expected late Feb); either the bud or the mesenchyme can be chimaeric in this system, and they can even be chimaeras with different exogenous cells.

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2. Materials

Observe good sterile technique when preparing and handling all instruments, solutions and hardware. Ensure that your supply of enzymes does not have sodium azide as a preservative.

2.1 Materials for the Edinburgh Method

1. Dissecting medium: Eagle's Minimum Essential Medium, Sigma cat # M5650.
2. Kidney culture medium (KCM): dissecting medium with Penicillin and Streptomycin (Sigma cat # P0781) and 10% foetal calf serum (Biosera).
3. Trypsin-EDTA 10X (Sigma cat # T4174) made up at 1X in phosphate-buffered saline, itself made from tablets (Sigma cat # P4417)
4. Glycyl-H1152 dihydrochloride (Tocris, Bristol, UK), made up to 1.25µM in KCM.
5. *Optional* for tracking test cells: Green CMFDA CellTracker dye (Molecular Probes, Invitrogen)
6. *Optional* for 'standard immunostaining': Methanol stored at -20°C, mouse anti-calbindin-D_{28K} (Ab9481, Abcam, Cambridge MA, USA), rabbit anti-laminin (L9393, Sigma), goat FITC anti-mouse (F0257/Sigma) and goat TRITC anti-rabbit (T5268, Sigma).
7. 5µm polycarbonate filter, Millipore.

8. Stainless steel culture grids (these are for supporting the culture filters at the liquid/gas interface. Make them from fine stainless steel mesh: cut the mesh into triangles about 1.5cm per side, and bend the corners down to make legs about 3mm high – the height is not critical, but having them very high wastes medium). We find it useful to force a pointed scissor blade into the mesh and to turn it, to make small 'holes' about 3mm across, across which pieces of filter will later be placed.
9. Microcentrifuge, tubes, 3.5mm Petri dishes, Cell strainer - 40μm (BD Falcon, Oxford, UK), Haemocytometer. *Optional* – fluorescence microscope (this is our favoured method for visualizing marked cells and immuno-stained antigens, but other methods such as sectioning and enzymatic staining are alternatives).
10. A *good* dissecting microscope. The difference that quality makes to the ability of an experimenter to place samples accurately on their culture filters cannot be over-emphasized. We use the Zeiss Stemi-2000 series; other excellent microscopes are available.

2.2 Materials for the Brisbane Method

1. Confluent Wnt4-expressing NIH3T3 cells (available from Andreas Kispert)
2. 100 μg/mL mouse collagen IV solution (BD Biosciences, 354233), and 0.65N HCl.
3. 0.4um polycarbonate filters (LabQuip Technologies, K04CP01300).
4. Dissecting medium: DMEM High Glucose (Invitrogen, 11995-073)
5. Scalpel blades
6. Microscopes: Dissecting microscope (Zeiss StemiSV6) for harvesting embryonic kidneys; Standard light microscope (Olympus CX31) for checking paraffin sections; Fluorescence microscope (Olympus BX51) for visualising immunofluroescence of resulting sectioned.
7. Accutase (Millipore, SCR005).
8. Phosphate buffered saline
9. Gilson P1000 and P200 pipettes (John Morris Scientific, F123601 and F123602)

10. *Optional* DiI or GFP-labelled test cells for analysis of renal potential. These may include cell lines, freshly isolated tissue or FACS sorted cell populations.
11. Shaking waterbath (Thermoline Scientific, TWBS-20)
12. Culture medium: DMEM supplemented with 100U/mL penicillin, 100g/mL streptomycin, 2nmol/L L-glutamine and 10% FCS. Standard tissue culture incubator at 37°C, 5% CO_2.
13. Stainless steel culture grids (these are for supporting the culture filters at the liquid/gas interface. Make them from fine stainless steel mesh: cut the mesh into triangles about 1.5cm per side, and bend the corners down to make legs about 3mm high – the height is not critical, but having them very high wastes medium).
14. Plasticware: 1.5 ml microcentrifuge tubes, 15ml Falcon tubes (Astral Scientific, I5570-02), 4-well dishes (Nunclon, D6789-ICS), 60 mm tissue culture dishes for dissection of kidneys (Thermotrace Ltd, TPP93060).
15. Cell strainer – 70 or 100µm (Becton Dickinson, 352350 or 352360)
16. Haemocytometer for quantitation of cells prior to reaggregation.
17. Benchtop centrifuge (Hettich Rotofix 32) for pelleting reaggregations in 15 ml tubes
18. Standard glass microscope slides; microtome for cutting sections.
19. *Optional* for 'standard immunostaining': 4% Paraformaldehyde stored at -20°C, alcohol series (70%, 80%, 90%, 100%), xylene, paraffin wax and microtome for paraffin embedding and sectioning. Antibodies: mouse anti-calbindin D_{28K} (Abcam, Ab9481), mouse anti-Six2 (Sapphire Biosciences, H00010736), mouse anti-Aquaporin1 (Millipore, #AB2219), rabbit anti-Pax2 (Zymed Laboratories, #71-6000), mouse anti-WT1 (DakoCytomation, #6F-H2 m3561), rabbit anti-pan-laminin (Sigma, L9393), rabbit anti-GFP (Sapphire Bioscience, #ab13970), Alexa Fluor 488 anti-rabbit (Invitrogen, A10254), Alexa Fluor 594 anti-mouse (Invitrogen , A10256).

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3. Methods

Both methods begin with kidney rudiments isolated from E11.5-E12.5 mouse embryos. Given the specialized natures of this volume, it is assumed that any reader is capable of performing this dissection, and a proper description of the process would require a chapter in itself. Such a chapter can be found in a previous volume of this journal (6).

3.1 The basic Edinburgh Method (see 3.2 for a refinement that results in a re-aggregate arranged around a single re-aggregated ureteric bud)

This method involves significant micro-manipulation of tissues, in open-air, in media that are intended to be buffered against 5% CO_2: pay close attention to the colour of the pH indicator in the medium while you work, and change medium if it begins to look significantly more alkaline than equivalent medium in a 5% CO_2 incubator. This is important – pH drift is bad for the cells (but so, alas, are all of the non-CO_2 buffers we have tried in an attempt to obviate the problem).

1. Isolate at least 10 fresh E11.5 metanephric rudiments in Dissecting Medium (defined in section 2.1).
2. Using a fine (pulled) Pasteur pipette, transfer the rudiments to a 3mm Petri dish containing Trypsin-EDTA solution and incubate them for 4 min at 37°C, 5% CO_2.
3. Using a glass Pasteur pipette, transfer the kidneys to a 3 mm Petri dish containing KCM. The precise volume does not matter much, but the presence of serum in the KCM is important for quenching (competing for) the trypsin-EDTA. Incubate the rudiments in this for 10min, 37°C.
4. This incubation is a good time to prepare culture filters for later use. Immerse a 5μm polycarbonate filter in KCM and cut it into squares about 5mm per side using a scalpel (rounded blades are less inclined to tear the filter). If necessary, small notches can be cut into filters as an identification code for samples, one notch being the filter for sample 1, 2 notches being for sample 2 etc. Cut one filter fragment into a triangle shape, for the control kidney. Place filter grids in fresh 3cm Petri dishes, and fill these dishes with KCM with H-1152 until the surface of the grids is just wet but the meniscus away from the grid still bends a little lower than the grid surface (excess KCM with H-1152 can be pipetted away to achieve this state of affairs). Place the pieces of filter on top of the grid (across the holes

in the mesh if you made them). Several pieces of filter can be put on one grid. Place the whole dish in the 37°C 5%CO_2 incubator until it is needed.

5. Place the organs in a 0.5 ml Eppendorf tube containing 200 microliters of KCM. Dissociate the organs by trituration – that is pipetting them up and down through a yellow Gilson tip adjusted to 100 microlitres. This step may need practice – too much violence results in shear stresses lethal to the cells, while too little fails to separate cells into a single-celled suspension.
6. Filter the resulting suspension through the cell strainer. Stain a sample of it with Trypan Blue and apply the stained cells to a haemocytometer both to check that (i) the cells are in a single-celled suspension, (ii) that they are alive (our results show an average of about 90% of cells being alive) and (iii) to measure their concentration.
7. Divide the cell suspension in the main, unstained sample, into aliquots of 8 x 10^4 cells, placing each aliquot in a 500µl microcentrifuge tube. Complete with KCM medium to obtain a solution of 150-200 microliters per tube.
8. *Optional*: add test cells to the suspensions. If these cells do not carry an intrinsic marker, eg GFP, they can first be stained with CellTracker according to the manufacturer's directions (a typical labelling procedure uses CellTracker at 4µM, but we recommend that a pilot experiment be performed to determine the maximum concentration of CellTracker compatible with viability of the cell type in question: in our experience this varies with both cell type and with batch of CellTracker,). The number of cells to be added must be determined by trial and error, typically by running a series of parallel experiments in which the test cells form increasing proportions of the final mix. When mixing labelled E11.5 kidney cells, we can use any proportion. In our experiments in which human amniotic fluid cells (hAFSC) were incorporated into kidney re-aggregates, we found the optimum ratio, for the best possible incorporation, was 10% of the final mix to be of hAFSC origin (5).
9. Centrifuge each suspension of cells for 2min at 800g to make a pellet.
10. Use a yellow pipette tip, on a pipette, to aspirate a little medium and then expel it, very gently, at each pellet to persuade the pellet to break free of the tube.

11. Remove the Petri dish containing the filters in the grid, prepared earlier at stage 4, from the incubator and place it at the stage of the dissecting microscope. Collect the pellets from their microcentrifuge tubes with a glass Pasteur pipette and release them *gently*, on to a square filter (one re-aggregate, one filter). Each will be just visible as a brownish 'stain'. Beware the risk of releasing so much medium that the re-aggregate is washed over the side of the filter.
12. Place the dish with all of its occupied filters in the incubator, taking great care to treat it gently (and warning other incubator users of the need not to slam doors etc). Incubate for 24h.
13. Remove the dish from the incubator, remove the grid with filters and place it in a new Petri dish. Quickly fill with fresh pre-warmed KCM with no H-1152 (continued presence of H-1152 prevents nephrons forming properly (1)). Return to the incubator for as long as desired (3 days is typical).
14. *Optional* – fix in 4% formaldehyde (made freshly from paraformaldehyde) in PBS for 30 min, wash in PBS and immerse in methanol for 10 min. The formaldehyde fix is necessary if CellTracker has been used, because fixation directly in methanol results in its loss. Methanol is needed for antibody access to the cytoplasm. If Cell tracker has not been used, fixation can be directly in ice-cold methanol. Use forceps to transfer filters to a bijou tube with PBS, and incubate them for at least 30 minutes at room temperature. Replace the PBS with a solution of primary antibody (each at 1/100, for the antibodies mentioned in section 2) and leave them overnight at 4°C. Wash in PBS during the next day, and incubate in secondary antibodies overnight at 4°C. Wash again in PBS, mount and observe.

3.2 The revised Edinburgh Method, for making a re-aggregate arranged around a single re-aggregated ureteric bud

This method consists essentially of two sequential rounds of the basic method, with some micro-dissection in-between. It is significantly more labour-intensive than the basic method, so should be used only when having a single ureteric bud is important.

1. Begin by setting up a conventional disaggregation and re-aggregation culture using the method described in 3.1 above. (*Optional:* If chimaeric ureteric buds are required, add labelled test cells as appropriate). Culture for 1 day in KCM with H-1152 and 3-4 days in plain KCM. This culture will contribute only the ureteric bud to the final experiment, and is referred to henceforth as the 'UB-donor culture'.
2. Prepare a new culture dish for the final culture (step 4 of section 3.1); plain KCM can be used for this.
3. Isolate a fresh batch of E11/5 kidneys. Under a dissecting microcope, use 25-gauge needles to pull the mesenchyme away from the ureteric bud without using enzymes.
4. Place the mesenchymes only in a Petri dish containing 0.5X Trypsin-EDTA for 2 minutes at 37 degrees in the incubator.
5. Place the mesenchymes in a Petri dish containing KCM and leave them for 10 minutes at 37 degrees in the incubator.
6. Place the mesenchymes in a 0.5 mL Eppendorf tube containing 200 microliters of KCM and triturate them using a yellow tip, the pipette being adjusted to 100 microliters, until they are a single cell suspension (filter them and verify their viability and that they are a suspension of single cells by the method of stage 6 in section 3.1). If it is critical to know that the final experiment is not contaminated by any ureteric bud cells carried over with this mesenchyme, keep a sample of the suspension and either immunostain it for a ureteric bud marker such as calbindin-D_{28K} or use RT-PCR.
7. *Optional* if the mesenchymal component of the final re-aggregate needs to be chimaeric, add test cells to the suspension of mesenchymal cells now.
8. Pellet the mesenchyme cells and recover the pellet using steps 9 and 10 of section 3.1; use about 10^5cells per reaggregate.
9. Recover the UB donor culture from the incubator, gently scrape it off its filter in KCM, using a gauge 25 needle, and under a dissecting microscope, cut out 'ureteric buds' (these can be distinguished from developing nephrons by their

large diameter and branched shape. The easiest to cut out are those that are only just branched). It is almost unavoidable that a few mesenchymal cells will come with the bud.

10. At the dissecting microscope, place one of these recovered 'ureteric buds' on each filter of the Petri dish prepared in step 2. These are almost invisible small when on a filter, so it helps to place them exactly in the middle of a hole in the underlying metal grid.
11. Pipette one reaggregate of fresh mesenchyme, prepared in step 6, on top of the ureteric bud.
12. Incubate for as long as required (3-4 days typical), and fix and stain as in step 15 of section 3.1.

Figure 1: In A, a re-aggregate produced by the basic Edinburgh method (section 3.1) cultured for a total of 7 days and stained with anti-calbindin and anti-laminin. Nephrons ('n') and numerous small 'ureteric buds', some of which are branched, are visible ('ubs'). In B, a re-aggregate produced by the refined Edinburgh method (section 3.2) and stained with anti-calbindin and anti-laminin. Note how the arrangement of the tissues is much more like that of a normal embryonic kidney, with nephrons ('n) arranged around a single branched ureteric bud ('ub'). Scale bar=100 microns.

3.3 The Brisbane Method

1. 24 hours prior to setting up reaggregate, seed $5x10^4$ Wnt4-expressing NIH3T3 cells in 50µl droplet of culture medium onto a 0.4µm polycarbonate filter (one filter per reaggregate). Float seeded filters in 1ml culture medium in 4-well dish. Incubate at 37°C, 5% CO_2.

2. Dilute 100µl concentrated collagen IV in 600µl 0.65N HCl. Pipette 100µl diluted collagen IV onto a 0.4µm polycarbonate filter (one filter per reaggregate). Incubate at room temperature for 1 hour before washing in 1x PBS.
3. Harvest embryonic kidneys in dissection media, allow 3-4 kidneys per reaggregate.
4. Collect kidneys into a pile, remove all media, and roughly mince kidneys using a sterile scalpel blade.
5. Collect minced kidneys into 1ml pre-warmed (37°C) Accutase. Digest in shaking waterbath at 37°C for 15 minutes, manually pipetting fragments every 5 minutes to aid digestion.
6. Centrifuge kidneys at 300rpm (15g), 5 minutes.
7. Resuspend pellet in 500µl pre-warmed (37^{o}C) culture medium, manually dissociating with a P1000 pipette to single cell suspension.
8. Pass cells through 100µm cell strainer to remove any remaining cellular clumps.
9. Count cells using haemocytometer.
10. If including labelled test cells in reaggregate, harvest cells into culture medium and obtain cell count using haemocytometer.
11. Into a 15ml tube, aliquot embryonic cells and the appropriate portion of test cells to give a final cell number of $4x10^5$ cells in 600 - 700µl culture medium. We usually would not add more than 10% test cells to a reaggregation.
12. Centrifuge at 2000rpm (650g), 2 minutes.
13. Culture dish set up: into an organ culture dish, layer one piece of triangular mesh, one filter with feeder cells (feeder cells facing upwards) and one collagen IV-coated filter (collagen layer upwards). Add enough culture medium to just reach the level of the filters, without making them too wet.
14. Using a P1000 pipette, carefully blow the reaggregate off the side of the 15 ml tube and draw up into the pipette.
15. Carefully pipette the reaggregate onto the top collagen IV filter. Let rest for 2 – 3 minutes then top up the medium level so the reaggregate is at a media-air interface.
16. Culture for 4 days at 37°C, 5% CO_2.

17. Keeping reaggregate on the collagen IV filter but discarding the filter seeded with feeder cells, fix in 4% paraformaldehyde for 10 minutes on ice, then wash in 1x PBS for 5 minutes, twice.
18. Process reaggregate into paraffin wax as described previously (Rumballe et al, 2008). Briefly, pass tissue through an ascending ethanol series (15 minutes in each of 70%, 80%, 80%, 90%, 90%, 100% ethanol) followed by 2 x 20mins in xylene, wax for 30 mins, 60°C, 15 mins, 60°C and 15 mins, 60°C.
19. Trim filter into square shape with 1 – 2 mm clearance around pellet using scalpel blader.
20. To embed, position filter/pellet on smallest edge with pellet on the side, such that pellet will be cut on sagittal plane.
21. Using microtome, cut into 7µm sections using the remaining filter as a guide. Regular checking on a standard light microscope is necessary to determine progress of sectioning.
22. After sectioning, sections can be stained and morphology assessed using standard histochemistry (haematoxylin and eosin or other stain) or assessed for contribution of test cells into reaggregation via standard immunofluorescence with the desired antibodies. Our preferred options when using GFP-labelled test cells is anti-GFP with a marker of either ureteric epithelium (calbindin 28kD or Pax2; Note Pax2 also marks the cap mesenchyme and developing tubules), cap mesenchyme (Six2 or WT1; note WT1 also marks early tubules and developing podocytes), basement membrane (pan-laminin; collagen IV) or a specific tubule marker (Aqp1 for proximal tubules).

Figure 2. Section analyses of chimaeric reaggregations. A. Histological section of embryonic kidney reaggregation showing evidence for a ureteric tip (black arrow), surrounding cap mesenchyme (white arrow) and developing nephron (arrowhead). B. Immunofluroescence of embryonic kidney reaggregation illustrating expression WT1 (red;

metanephric mesenchyme and developing nephrons) and pan-laminin (green; epithelial basement membranes). C-E. Immunofluorescence of 50:50 reaggregation between wildtype and GFP+ embryonic kidney. GFP; green. WT1, red. F,G. Reaggregations of 50:50 wildtype embryonic kidney and cells FACS-sorted from the embryonic kidneys of Sal1-GFP mice demonstrating the application of the method to test renal potential of an isolated cell population. Sal1GFP+ cells; green. F, Calbindin 28Kd (ureteric epithelium), red. G. WT1, red.

4. Notes

1. Do not leave the cells for too long in suspension. The longer the cells will stay in suspension the more cell death will be observed.
2. During the dissociation step (step 3.5 of section 3.1) do not be too gentle. If the kidneys are not pipetted repeatedly and fast enough they will stick to the pipette tip and the dissociation will be inefficient.
3. Observe good sterile techniques to avoid culture contamination. Place metal grids and forceps in ethanol, when they are not used, and sterilize them by burning the remaining ethanol covering them before use.
4. If cells are left too long in a microcentrifuge tube, they will start to attach to the walls of the tube and the centrifugation step will not be efficient. Mix the cell suspension before centrifugation.
5. After centrifugation, the pellet should have a nice round shape at the bottom of the tube. The line along the walls corresponds to the presence of cellular debris and can mean that the cells were dissociated too strongly or that they were left for a long time in suspension.

Acknowledgements

This work was supported by NC3Rs grant G0700480, EU Star-t-Rek network FP7 223007, Australian Stem Cell Centre P067 and ID631362, National Health and Medical Research Council of Australia. We thank Caroline Hendry, Institute for Molecular Bioscience, for assistance with Figures.

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